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Exploring adult hippocampal neurogenesis using optogenetics

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EXPLORING ADULT HIPPOCAMPAL NEUROGENESIS USING OPTOGENETICS

by

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ABSTRACT

In the 1980s, it was widely accepted that new neurons are continuously generated in the dentate gyrus of the mammalian hippocampus. Since its acceptance, researchers have employed various techniques and behavioral paradigms to study the proliferation, differentiation, and functional role of adult-born neurons. This literature thesis aims to discuss how optogenetics is able to overcome the limitations of past techniques and provide the field with new insights into the functional role of neurogenesis. We will review the current knowledge on both adult hippocampal neurogenesis and optogenetics, present representative studies using optogenetics to investigate neurogenesis and discuss potential limitations and concerns involved in using optogenetics.

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ABBREVIATIONS

AAV	Adeno-associated virus
abGC	Adult-born granule cell
Arch	Archaerhodopsin
BMI	Bicuculline methiodide
BrdU	5'-bromo-2'-deoxyuridine
ChR2	Channelrhodopsin-2
DCX	Doublecortin
DG	Dentate gyrus
EF1α	Elongation Factor 1 alpha
ER	Estrogen receptor
fEPSP	Field excitatory postsynaptic potential
GCL	Granule cell layer
GPCR	G protein-coupled receptor
i.p.	Intraperitoneal
Kyn	Kynurenic acid
LED	Light-emitting diode
LTP	Long-term potentiation
LV	Lentivirus
MAM	Methylazoxymethanol acetate
mRFP1	Monomeric red fluorescent protein 1
MWM	Moris water maze

NSC	Neural stem cell
PSC	Postsynaptic current
RV	Retrovirus
SGZ	Subgranular zone
SVZ	Subventricular zone
TBS	Theta burst stimulation
tk	Thymidine kinase
TM	Tamoxifen
VSVg	Vesicular stomatitis Indiana virus' G protein
wpi	Weeks post injection

INTRODUCTION

The phenomenon of adult hippocampal neurogenesis, the formation of new neurons in the hippocampus of adult brains, was first discovered in rodents in the 1960s using [(3)H]-thymidine autoradiography (Altman and Das, 1965). However, its occurrence in nonhuman primates and humans was not widely accepted until the 1980s, when the development of confocal imaging technology and novel immunohistological co-labeling techniques allowed scientists to more accurately label and identify the adult-born neurons (Miller and Nowakowski, 1988; Taupin, 2007)

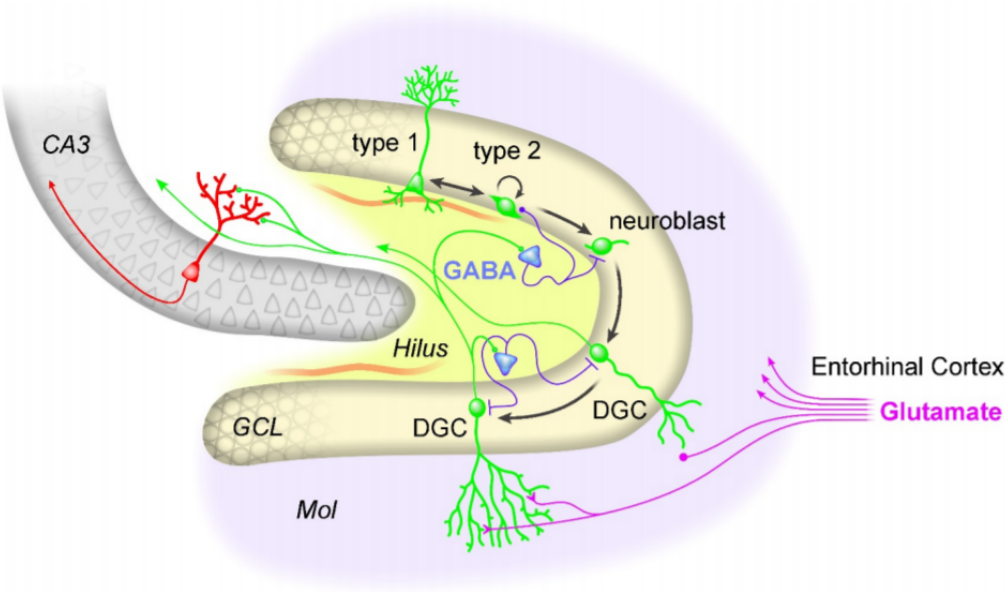

Figure 1. Hippocampus circuitry and the development of NSCs into mature DG neurons. (Figure taken from Mu and Gage, 2011)

It is now universally accepted that neurogenesis continues throughout adulthood in at least two areas of the mammalian brain, the subgranular zone (SGZ) of the dentate

gyrus (DG) in the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Gage, 2002). The SGZ is made up of tissue located between the granule cell layer of the DG and the hilus of the hippocampus. While the majority of new neurons from the SVZ migrate to the olfactory bulb and become interneurons, those from the SGZ migrate into the granular layer of the DG and become granule neurons (Kee et al., 2007; van Praag et al., 2002). The DG receives input from the entorhinal cortex and sends the signal through its axonal projection into the CA3 region of the hippocampus (Figure 1). Thus, it is implicated in many of the hippocampus' roles, such as learning, the formation of new memories and spatial navigation tasks (Jonas and Lisman, 2014; Gonçalves et al., 2016). Consequently, research efforts in the field has been focused on, among other things, understanding the underlying mechanism by which adult-born DG neurons incorporate into the hippocampal circuitry, how these neurons are regulated by intrinsic and extrinsic factors, as well as the potential roles that they play in learning and memory (Aimone et al., 2014).

Current methodologies for testing the functional significance of adult neurogenesis in rodents rely heavily on completely ablating neurogenesis and measuring its impact on performance in a learning or memory task. These techniques range in specificity and invasiveness; from using gamma irradiation to preferentially kill any rapidly dividing cells in the brain, to creating transgenic rodent models which kills only new neurons in the adult brain upon consumption of Ganciclovir-laced chow (Clark et al., 2008; Hamilton et al., 2015; Mustroph et al., 2015). A common problem to these methods of removing new neurons from the circuit is one of compensation, whereby pre-existing

neurons can extend their function to compensate for the loss of new neurons. This could mask the real function of adult-born DG neurons and may lead to conflicting results. Thus, the development of a technique or combination of techniques that can manipulate the activity of adult-born neurons with spatial and temporal precision would allow scientists to more accurately study the functional role of adult-born neurons.

Optogenetics is a technique which allows for both temporal and cellular specificity in manipulating the activity of genetically modified mammalian tissues. The technique began to take shape in 2005, when Boyden et al. (2005) was able to transfect a culture of hippocampal neurons to localize expression of channelrhodopsin-2 (ChR2), a microbial-derived light-gated cation channel, in its membrane. The group was able to cause depolarization in the transfected neuron within 50 μs of exposure to blue light. This experiment demonstrated the potential for using microbial opsins and light to manipulate neuronal activity. However, due to technological constraints, it wasn't until 2009 that researchers were able to take full advantage of optogenetics' ability to spatiotemporally control neuronal activity and manipulate behavior (Deisseroth, 2015).

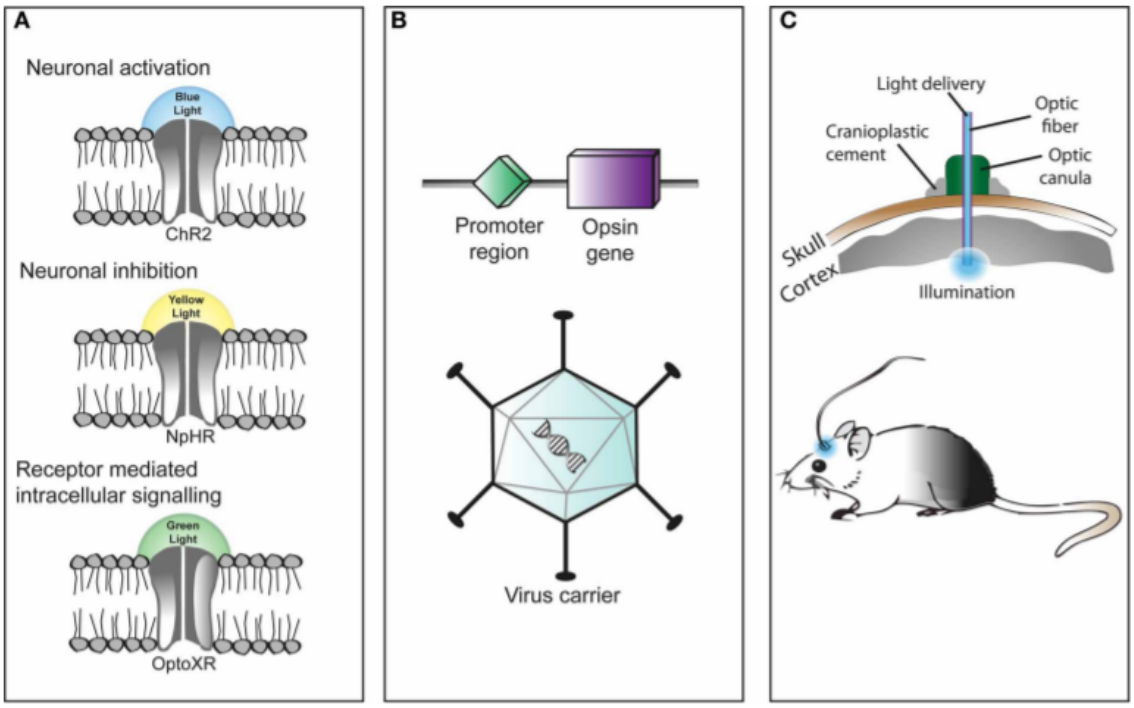

Figure 2. The three core components of optogenetics. (A) opsins, (B) genetic targeting of opsin gene (e.g. viral-mediated delivery), and (C) optics (i.e. light-delivery technology). (Taken from Claudia Pama, Colzato, and Hommel, 2013)

Optogenetics is composed of three core components: 1) microbial opsins (e.g. ChR2), 7-transmemberane, light-responsive proteins, 2) genetic targeting, which allows for opsin expression in specific neuronal phenotypes and 3) optics, light-delivery technology which provides temporal and somewhat spatial precision (Deisseroth, 2015). In addition to providing the technique with both spatial and temporal precision, the many possible combinations of the components set optogenetics up to be versatile and adaptable. For example, choosing to deliver ChR2 in a cell-specific viral vector might be advantageous if robust expression of ChR2 is needed in a localized area of the brain, but a transgenic mouse line expressing ChR2 under the control of a cell-specific promoter might be better if the targeted cell type can be found in more than one area of the brain.

Over the past decade or so, the rapid evolution of opsin engineering, targeting methods, and optic technology has allowed optogenetics to take center stage in neuroscience research. The field of adult hippocampal neurogenesis is no exception as researchers have been able to discover novel and impactful findings within the field by using optogenetics techniques (Toni et al., 2008; Gu et al., 2012; Ramirez et al., 2013; Danielson et al., 2016)

This literature thesis aims to provide evidence of optogenetics' potential to further our understanding of adult hippocampal neurogenesis. In doing so, we will review the current knowledge on adult hippocampal neurogenesis and optogenetics, as well as discuss the findings of representative papers from the past decade in which researchers have used optogenetics to explore adult hippocampal neurogenesis.

Adult Hippocampal Neurogenesis

Development of adult-born neurons in the DG

As discussed previously, neurogenesis occurs in the SGZ of the DG, because it contains a microenvironment that is suitable for neuronal development, which is referred to as the neurogenic "niche". The neurogenic niche contains the necessary neural stem cell (NSC) population, secreted growth factors such as fibroblast growth factor-2 (FGF-2), and cell-cell contact (e.g. microglia and astrocytes) for adult neurogenesis to occur (Gage, 2000; Song et al, 2002; Yoshimura et al., 2003; Gonçalves et al, 2016).

The developmental process of adult-born hippocampal neurons from NSCs into granule cells takes about 7 weeks and is similar to that of the embryonic hippocampal neurons. NSCs are defined by their ability to both self-renew and differentiate into specialized cells. During this process, the cells go through 3 distinct phases: cell proliferation, neuronal differentiation, and cell survival (Figure 3) (Aimone et al., 2014).

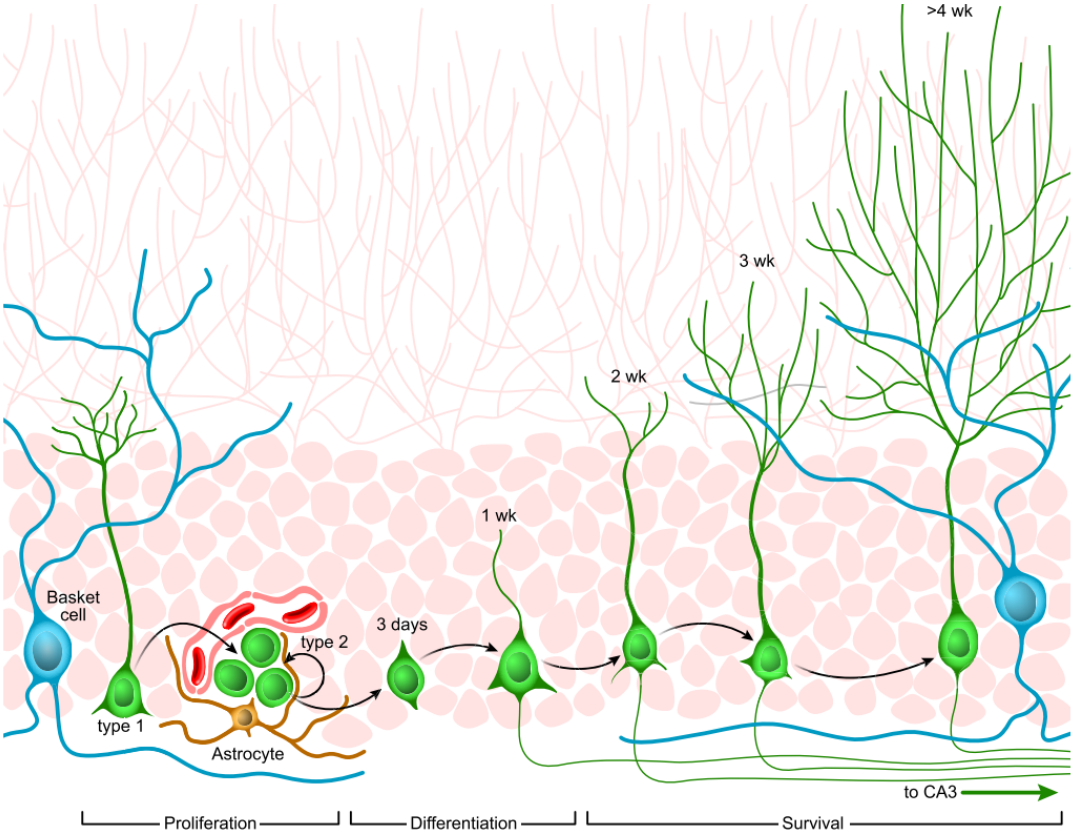

Figure 3. The development of dentate gyrus granule cells. (Figure taken from Aimone et al., 2014)

At the start of neurogenesis, NSCs called Type 1 radial glia-like cells come out of their quiescent state and undergo cellular division into the highly proliferative intermediate progenitor cells (type 2 cells). As such, the type 2 cells' main function is to

proliferate while also giving rise to neuroblasts (type 3), which goes on to differentiate into granule cells (Figure 1). At the level of type 2 cells, each cell either undergoes neuronal differentiation or becomes an astrocyte. Type 2 cells who are fated to become granule cells must express specific transcriptional factors (e.g. NeuroD1, Prox1, and Tlx) and genes [e.g. nestin and doublecortin (DCX)] (Beckervordersandforth, 2015) (Figure 4).

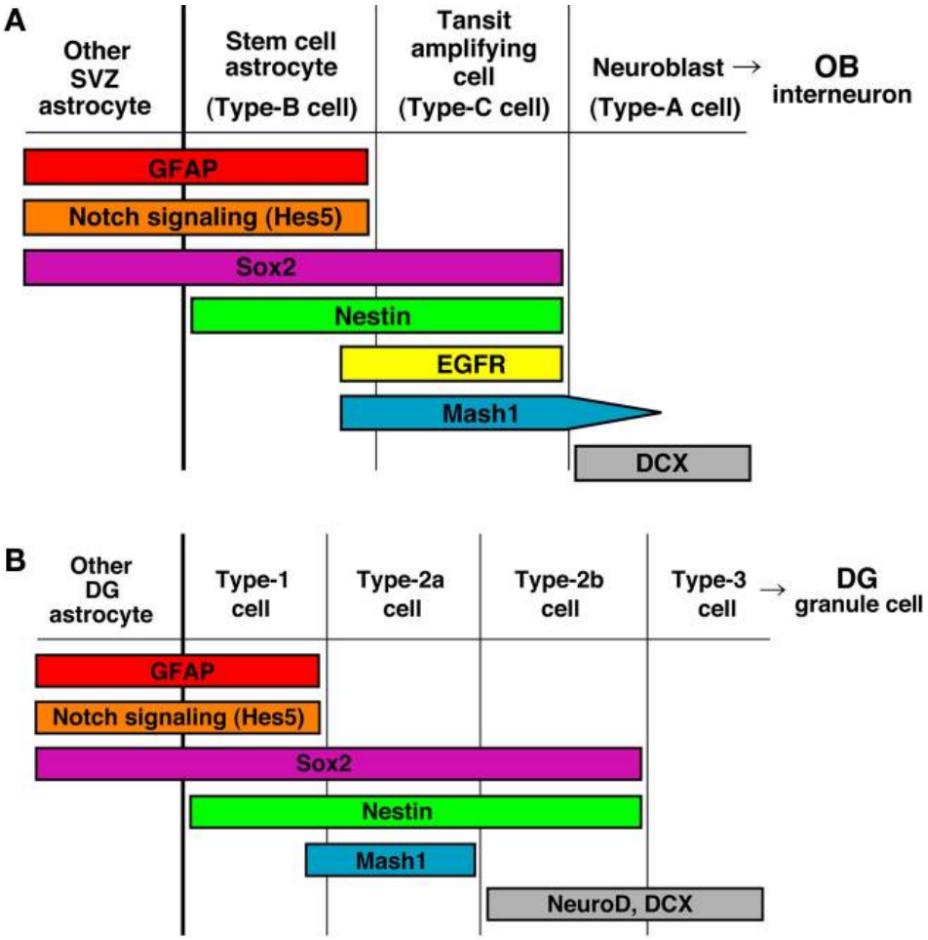

Figure 4. Adult-born neuron-specific transcriptional factors and genes expression. (Figure taken from Imayoshi, Sakamoto and Kageyama, 2011)

An essential phase in the maturation of the adult-born neurons is the cell survival phase. This phase happens around the time type 3 cells start expressing postmitotic markers such as NeuN and Calretinin (Brandt et al., 2003). Most of the type 3 cells undergo apoptotic elimination during this phase and the few that survive experience further morphological and physiological changes before becoming a fully integrated granule cell. Two major morphological changes that occur in the surviving type 3 cells are axonal growth and dendrite formation. The elongation of the cell's axon to the CA3 region precedes the development of dendritic spines which projects out towards the molecular layer of the hippocampus through the hilus. As the axon passes through the hilus, it forms synapses with interneurons before finally synapsing onto the pyramidal cells of the CA3 region (Zhao et al., 2006).

An important component to the maturation and proper integration of adult-born neurons into the DG circuitry is synaptic input from the surrounding neurons. More specifically, at around a week old, the type 3 cells begin to receive GABAergic input from the surrounding neurons. However, the cells' response to the input is excitatory and the input drives them to neuronal maturation (Ge et al., 2006). At around 3 weeks of age, the cell's dendrites start receiving regular glutamatergic input from the entorhinal cortex while they finish extending their axons to the CA3. A unique property of immature (i.e. between 4-6 weeks old) adult-born type 3 cells is that they express NMDA receptor subunit NR2B29, which results in a lower threshold for long-term potentiation (LTP) and a higher LTP amplitude. The hyper-excitable and plastic nature of the cell allow them to make distinct contributions to learning and memory when compared to mature granule

cells (Ge et al., 2007). Finally, once the cell is the hippocampal subregions surrounding the DG (i.e. molecular layers, granule cell layers and hilus) send local GABAergic input to the cell, which now results in an inhibitory response due to the increasing number of inhibitory synapses in the cell (Li et al., 2012).

Functional role of adult hippocampal neurogenesis

The hippocampus is a brain region that is critical for declarative memory (e.g. what, when and where) and thus the adult-born neurons are thought to contribute to hippocampal-dependent learning and memory. Data from numerous studies seem to suggest that adult neurogenesis is associated with "pattern separation", which is a process that differentiates similar information received by the DG into different outputs sent to the hippocampus (Gonçalves, Schafer, and Gage, 2014). Behaviorally, pattern separation can be tested in rodents by using spatial discrimination tasks such as the radial-arm maze, fear context discrimination, and the two-choice discrimination task. Although neurogenesis ablation studies have found some inconsistent data regarding its effects on hippocampal-dependent tasks, studies which looked at the effect of increased or ablated neurogenesis on discrimination task performance has found a strong connection between the two (Clelland et al., 2009; McTighe et al., 2009; Creer et al., 2010; Deng et al., 2010; Sahay et al., 2011; Nakashiba et al., 2012)

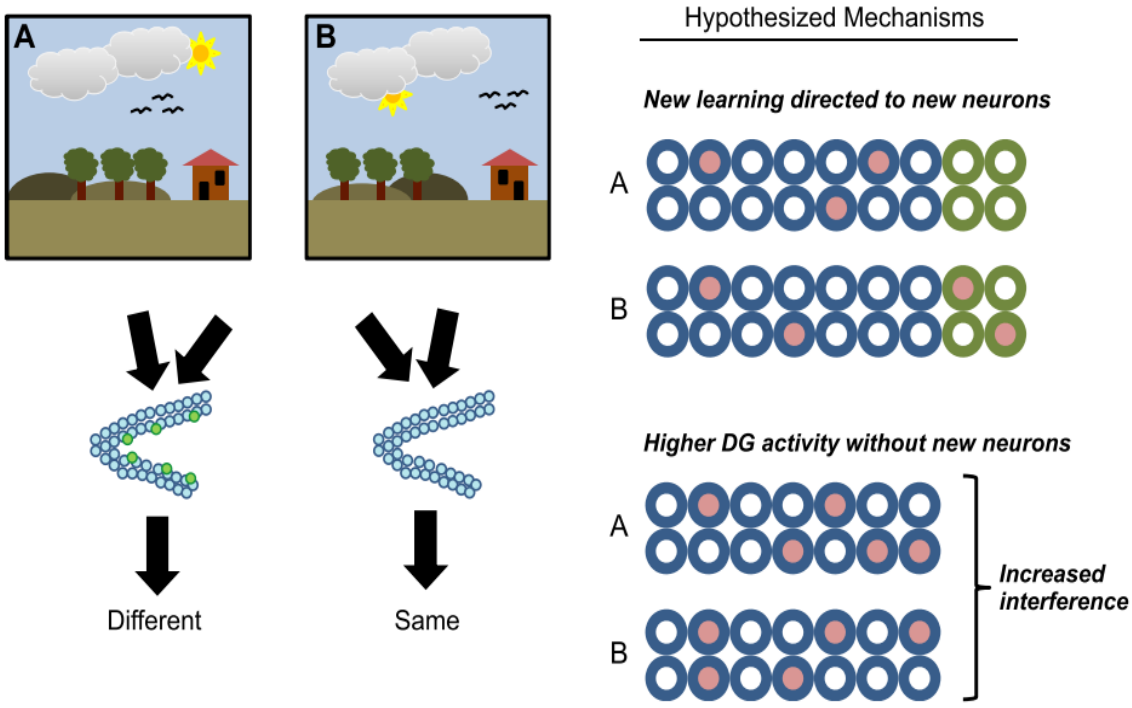

Figure 5. A model of the pattern separation theory for adult hippocampal neurogenesis. *Left*: DG with adult-born neurons will encode picture A and B distinctly and produce separate outputs, unlike DG without adult-born neurons. *Top-right*: Addition of new neurons provide more space for new learning during encoding of inputs. *Bottom-right*: Lack of new neurons result in higher activity in the old neurons, resulting in increased interference. (Taken from Aimone et al., 2014)

Although we have substantial behavioral evidence for the effect of adult neurogenesis on pattern separation, the exact mechanism behind the phenomenon is still under debate. Aimone et al. (2014) proposes that neurogenesis reduces interference between similar memories by providing a separate storage space (i.e. the new immature granule cells) for novel information within the new memory (Figure 5). In this manner, new memories and old memories are encoded distinctly within the DG and are sent as separate outputs to the hippocampus. If there were no new neurons, the mature granule cells would have to increase their baseline activity in order to try and encode all of the

new memory. This leads to an overlap between new and old memories, thus reducing pattern separation (Aimone et al., 2014).

Interestingly, in a computational model of the DG system made by Aimone et al. (2009), in addition to the pattern separation phenomenon, they also found that dissimilar inputs led to outputs that were inversely related to the degree of neurogenesis. In other words, in DG system with a high degree of neurogenesis, dissimilar inputs actually led to outputs that were similar to one another. When they removed the immature neurons from analysis, they found that the outputs were no longer overlapping. They speculated that immature granule cells, unlike the mature granule cells, have a harder time distinguishing between dissimilar inputs and would form associations between them. This proposed function of immature granule cells, which they named "pattern integration", is supported by their unique electrophysiological properties. Between 4-6 weeks of age, immature granule cells are more excitable, have a lower LTP threshold, and a higher LTP amplitude than mature granule cells (Li et al., 2012). This period is known as a "critical period", a period when the immature cells will respond to a broad range of stimuli and can quickly reinforce active connections (Gonçalves et al., 2016). Thus, they are less selective and can fire in response to inputs that are dissimilar, as opposed to the mature granule cells which are very selective in their firing.

Currently, the consensus on the function of adult hippocampal neurogenesis is that it contributes to learning and memory. As to how it does so is still up for debate. One widely accepted theory is that adult-born neurons support the DG's role in behavioral

pattern separation tasks, as supported by a growing body of data (Gonçalves et al., 2016). However, we are currently relying on computational models to try and understand its mechanism instead of studying it *in vivo*. Recent technological developments such as optogenetics will allow for spatial and temporal control of cellular activity. When combined with other techniques such as *in vivo* 2-photon calcium (Ca2+) imaging, it could be used to observe adult-born neuron activity during spatial discrimination tasks in real time. This is one of many examples of how new technology could be used to deepen our understanding of the mechanisms behind the development and function of adult-born neurons.

Methodologies in the field of neurogenesis

The field of neurogenesis research gain its footing with the development of 5'-bromo-2'-deoxyuridine (BrdU) labeling technique as a way of labeling actively-dividing cells. BrdU is a thymidine analog which incorporates itself into the cell during its S-phase (Hoshino et al., 1986). By combining confocal imaging together with immunohistological co-labeling techniques, scientists were able to visualize adult hippocampal neurogenesis from rodent brain samples (Taupin, 2007). The techniques used to study the physiology and function of neurogenesis grow more complex as new technologies are made available. We will discuss BrdU-labeling and other techniques in more detail below.

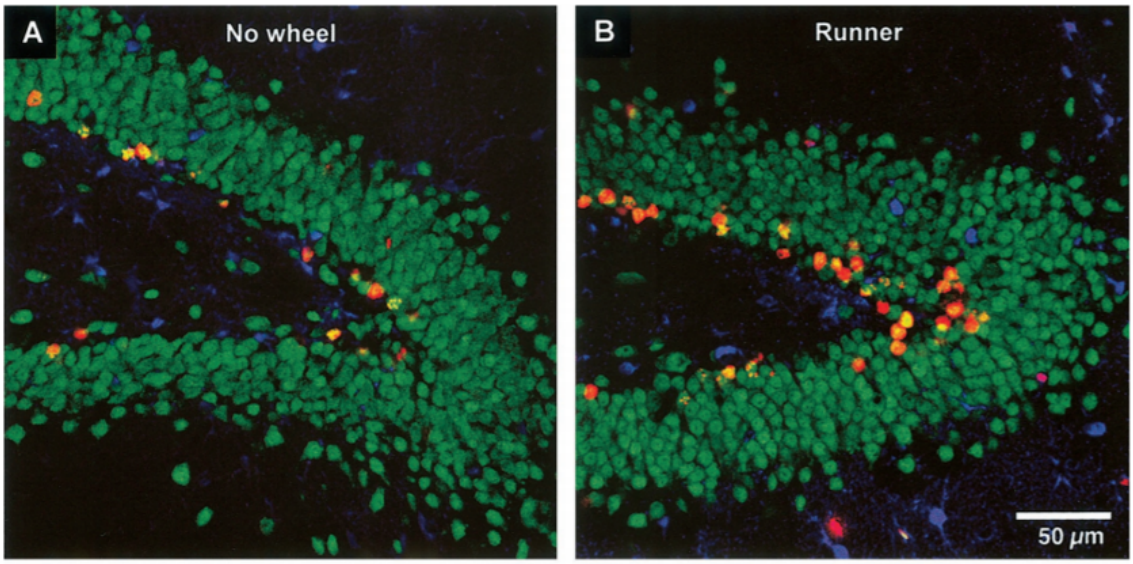

Figure 6. Confocal image of mice DG stained for BrdU, NeuN and S100. A triple-label (BrdU, NeuN, and S100) immunohistochemical assay was done on the brain samples of a mouse housed without a running wheel (A) and with a running wheel (B). Red cells are BrdU+, green cells are NeuN+, blue cells are S100+, purple cells are both BrdU+ and S100+, yellow cells are both BrdU+ and NeuN+. (Taken from Rhodes et al., 2003)

To quantify neurogenesis, scientists use a BrdU injection regimen on rodents to label the newly-dividing cells (i.e. mainly type 2 cells) in the SGZ of the hippocampus. An immunohistochemical assay is then done on their brain samples to visualize the BrdU+ cells using confocal imaging. The samples are usually also co-labeled with neuronal markers such as NeuN as not all BrdU+ cells are fated to be neurons (Figure 6). Thus only cells that are both BrdU+ (i.e. newly-divided) and NeuN+ (i.e. a neuron) will be quantified. Limitations of BrdU-labeling is that the compound is only active in the animal 2 hours post-injection and may label cells undergoing DNA repair (Taupine, 2007). An alternative method of measuring proliferative activity in the SGZ is by detecting for Ki67+ cells. Ki67 is a nuclear protein expressed in all phases of the cell

cycle except for the resting phase and thus, is a reliable marker for cells that re-enter the cell cyle (e.g. type I and type II cells). Unlike BrdU, Ki67 does not label cells undergoing DNA repair. However, it is also not suitable for detecting mature adult-born neurons as mature neurons have already exited the cell cycle (Kee et al., 2002)

A technique that has been used to characterize the survival, migration and morphology of adult-born neurons in the DG is by injecting rodents with a fluorescent protein-carrying retrovirus (Tanaka et al., 2004; Ge et al., 2006; Farioli-Vecchioli et al., 2008). Retroviruses (RVs), unlike adeno-associated viruses (AAVs) and lentiviruses (LVs), have the unique property of only infecting actively-dividing cells (i.e. type 2 cells). RVs can be a powerful tool for visualizing and studying the development of adult-born DG neurons. One method is by constructing an RV containing a fluorescent protein gene under the control of an immature neuron-specific promoter, which would allow researchers to track and visualize type II cells as they differentiate and mature into fully integrated DG granule cells (Enikopolov et al., 2015) (Figure 7).

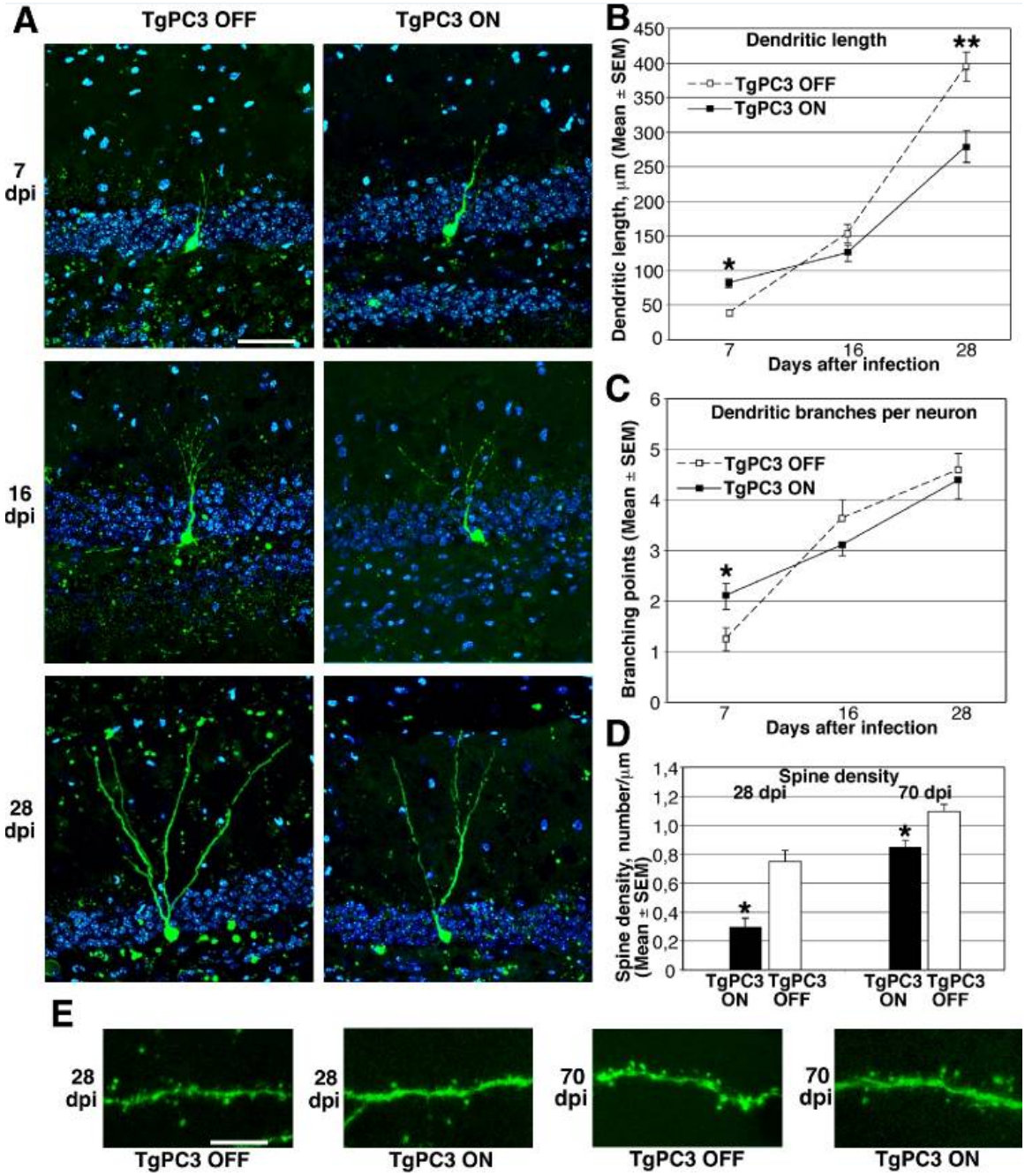

Figure 7. Morphology of adult-born neurons in TgPC3 OFF vs TgPC3 ON. The neurons were labeled with GFP-containing retrovirus. Mice with TgPC3, a pro-differentiation gene, ON displayed shorter dendritic length and fewer dendritic branches than the control mice at 16 and 28 days post injection (Taken from Farioli-Vecchioli et al., 2008)

The most common method of studying the function of hippocampal neurogenesis is to ablate neurogenesis observe its effects on cognitive performance. Early methods of neurogenesis ablation relied on the fact that the proliferative type II cells are more sensitive to neurotoxins [e.g. methylazoxymethanol acetate (MAM)] and irradiation than fully mature neurons. Unfortunately, these methods have been known to be nonspecific, cause inflammation and have side effects to the general health of animals, such as reduced body weight and fur loss (Dupret et al., 2005).

A more specific and thorough method of ablating neurogenesis involves the creation of transgenic mouse models which expresses apoptosis-inducing genes driven by neurogenesis-specific promoters (e.g. nestin). Many different animal models have been developed to achieve this goal. For example, the nestin thymidine kinase (tk) mouse model is able to induce cell death in actively-dividing cells only when they are fed with ganciclovir-containing chow. The protein tk from herpes simplex virus is able to phosphorylate ganciclovir into an active nucleotide analogue which disrupts the cell's DNA replication and lead to apoptosis (Hamilton, 2015; Mustroph, 2015). Another example is the nestin–CreER/NSE–DTA (nestin– CRE recombinase-modified estrogen receptor/neuron-specific enolase 2–diphtheria toxin fragment A) mouse model. This mouse model makes use of an inducible site-specific Cre-lox recombination system which can be used to delete, insert, or translocate genes at specific DNA sites. When the mouse is fed with an estrogen analogue such as Tamoxifen (TM), the Cre protein, fused to the hormone-binding domain of the estrogen receptor (ER), is able to enter the nucleus and delete the loxP-flanked or "floxed" STOP codon from the DNA (Metzger et al.,

1995). This leads to the expression of diphtheria toxin, which leads to protein synthesis arrest and subsequent cellular death (Imayoshi et al., 2008) (Figure 8).

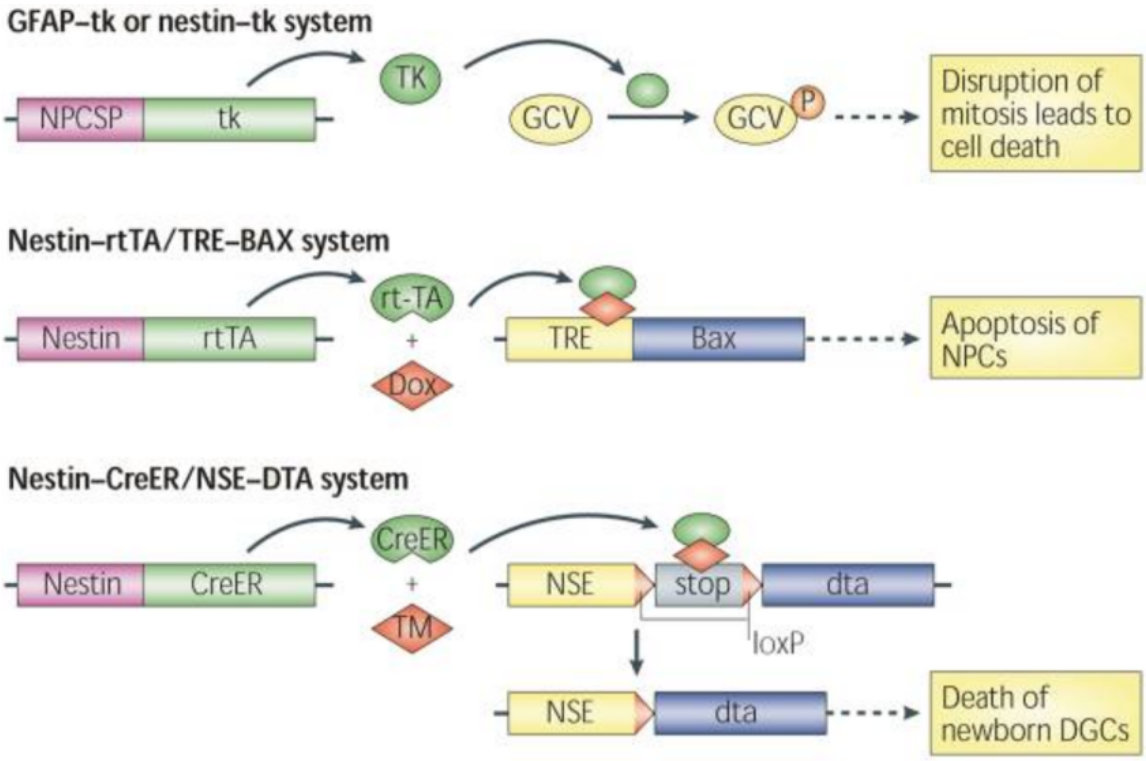

Figure 8. Sample transgenic rodent models for ablating neurogenesis. (Taken from Deng et al., 2010)

While these methods have been successful in ablating neurogenesis, the reported effect on hippocampal-dependent tasks have been somewhat inconsistent. A review done by Deng et al. (2010) highlights that the data can be inconsistent among studies using similar behavioral paradigms. For example, in regards to performance on contextual fear conditioning, a hippocampus-dependent task, poor performance has been seen in studies which use irradiation techniques (Winocur et al., 2006; Warner-Schmidt, Madsen, and

Duman, 2008) and in studies using two different transgenic lines, a Glial fibrillary acidic protein-tk line and a nestin–CreER/NSE–DTA line (Saxe et al., 2006; Imayoshi et al., 2008). However, no difference in performance was detected in studies using different ablation techniques such as MAM treatment and a transgenic nestin-tk line (Shors et al., 2002; Deng et al., 2009).

An explanation for these contradictory findings is that they are brought about by differences in experimental and behavioral paradigm designs (e.g. experimental timeline, duration of pre-training procedures). Additionally, it is important to note that a lack of abGCs at different maturation stages may manifest in different behavioral phenotypes. For instance, type 1 cells at 1 week of age does not receive any synaptic input from the surrounding cells and so its death would probably not affect learning and memory. However, a loss of immature adult-born type 3 cells with their enhanced excitability and plasticity would have a higher chance of adversely affecting learning and memory. Finally, there is also the problem of compensation, whereby the brain senses the loss of neurogenesis and compensates by extending the function of pre-existing neurons (Mustroph et al., 2014). The brain's compensatory mechanisms may mask the real function of adult-born DG neurons during cognitive performance.

Since the introduction of optogenetics in 2005, the technique has developed and expanded for use in neuroscience research. In using it to study neurogenesis, researchers would introduce an opsin, either through a viral vector or a transgenic mouse line, into the adult-born neurons in order to temporarily and precisely control its activity during

behavioral tasks. A method such as this would solve the potential problem of the brain's compensatory mechanisms.

Optogenetics

Development of optogenetics

As previously mentioned, the method of using opsins to control neuronal activity began with a simple experiment in which ChR2-expressing hippocampal neurons were depolarized with pulses of blue light. This paper was groundbreaking because it showed that: 1) microbial opsins, such as ChR2, can be stably expressed in neurons and locally targeted to the neuronal membrane, 2) ChR2 could be used to drive neuronal depolarization, and 3) ChR2's actions can be controlled by pulses of blue light with milliseconds timescale temporal resolution. That same year, different groups would soon follow in demonstrating the use of ChR2 *in vivo* or *in situ* to manipulate the activity of neurons (Li et al., 2005; Nagel et al., 2005).

In 2007, Adamantidis et al. demonstrated that it was possible to target a population of neurons (e.g. hypocretin neurons in the hypothalamus) with high specificity and to casually link behavior with activation of said neurons (i.e. activation of hypocretin neurons using fiber-optic hardware led to wakefulness from a sleeping state). This ability to target specific neurons or population of neurons was further advanced by the development of cell-targeting opsin viruses, transgenic opsin mouse lines, and projection targeting (Atasoy et al., 2008; Gradinaru et al., 2009; Sohal et al., 2009; Hägglund et al., 2010). By 2009, advancements in the 3 core components of optogenetic made it the go-to

method for researchers to spatiotemporally manipulate neurons and control behavior; as evidenced by the exponential increase in PubMed publications searchable by the keywords “Halorhodopsin”, “channelrhodopsins”, and other variations of optogenetics (Figure 9).

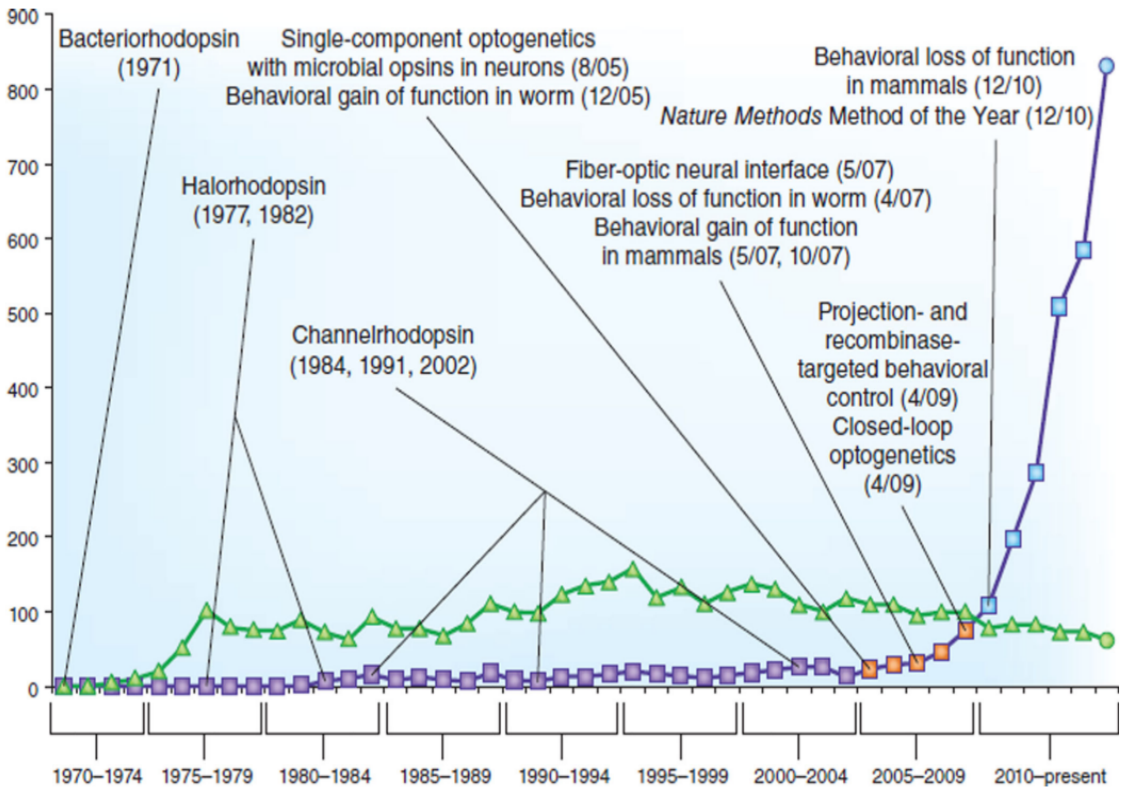

Figure 9. Publication timeline of optogenetics. The green triangles represent the number of papers searchable in PubMed by “bacteriorhodopsin” per year, while the squares represent papers searchable by the keywords “halorhodopsin”, “channelrhodopsin”, or other variations of optogenetics. The PubMed search was done on 1 July 2015(Taken from Deisseroth, 2015)

Opsins

Opsins are a family of 7-transmembrane light-sensitive proteins. They can be divided into 2 superfamilies: microbial opsin (type I) and animal opsin (type II). When opsins are bound to a form of Vitamin A called retinal, they become a functional unit called rhodopsins. When bound, retinal allows the opsin to respond to light as it isomerizes in response to photons and starts a series of conformational changes within the opsin that ends with its intended activity. While animal opsin binds to the 11-cis configuration of retinal that photoisomerizes into the all-trans configuration, microbial opsin binds to the all-trans configuration of retinal that photoisomerizes into the 13-cis configuration. Another difference is that, unlike animal opsin's retinal, microbial opsin's retinal does not dissociate from the opsin protein upon photoisomerization and would, instead, thermally revert back into the all-trans configuration for another round of photon activation (Fenno, Yizhar, and Deisseroth, 2011). Serendipitously, it was discovered that the mammalian brain already has sufficient levels of all-trans retinal for opsins to work (Zhang et al., 2006).

Metazoan opsins are present in higher eukaryotes and are responsible for the mechanism underlying vision (Shen et al., 2013). They are G protein-coupled receptors (GPCRs) and as such, are associated with the control of intracellular GPCR signaling pathways. Airan et al. (2009) and Oh et al. (2010) were able to develop light-sensitive GPCRs modified with alpha-1, beta-2 receptor and 5-HT1a receptors that are coupled to Gq, Gs, and Gi/o signaling pathways respectively. These proteins are called OptoXRs,

where X refers to the particular signaling pathway (e.g. Optoα1AR = α1 adrenergic receptors)

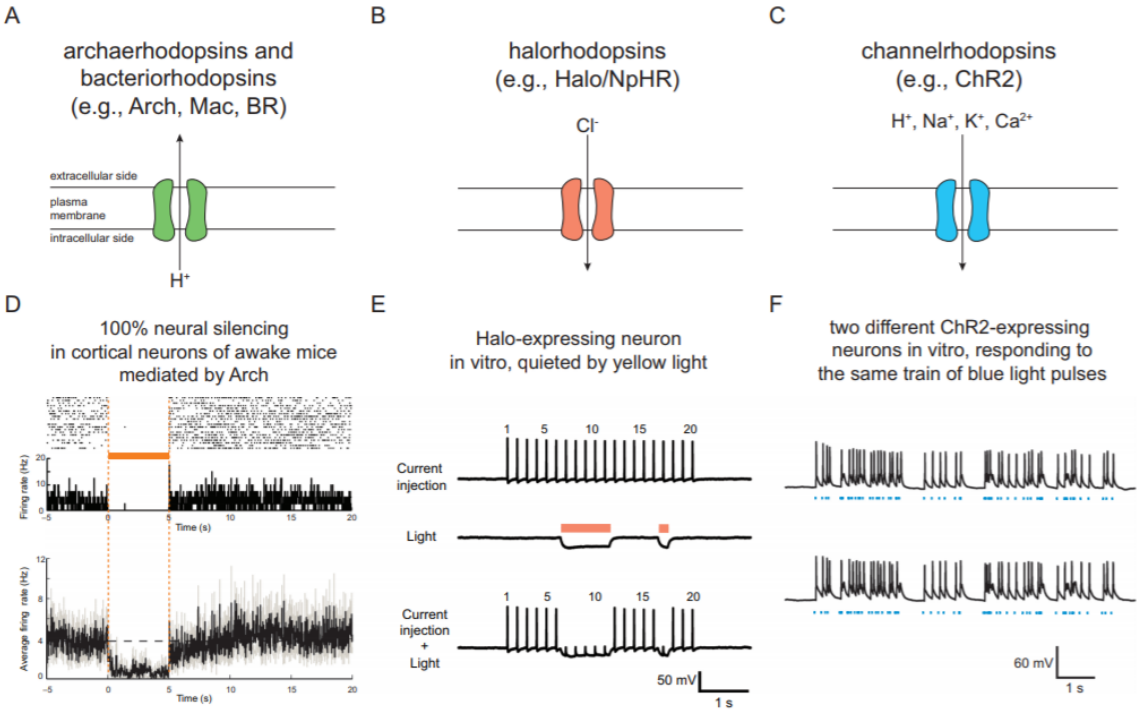

Figure 10. Microbial opsin family. (A,D) Archaerhodopsin and Bacteriorhodopsin pumps protons out of the cell and causes hyperpolarization, (B,E) Halorhodopsin pumps chloride ions into the cell and causes hyperpolarization, (C,F) Channelrhodopsin is a nonspecific cation channel, allowing cations to flow into the cell and causing depolarization (Taken from Boyden, 2011)

Microbial opsins are composed of different subfamilies (e.g. bacteriorhodopsin, Halorhodopsin and channelrhodopsin) and are found in prokaryotes, algae, and fungi (Supdich 2006). Unlike metazoan opsins, microbial opsins combine the task of light sensation with ion flux, manipulating its membrane potential and controlling the neuron's excitability (Figure 10). In regards to optogenetics, microbial opsins can be categorized as either excitatory or inhibitory. For example, ChR2 is an excitatory light-gated

nonspecific cation channel that lets in cations in response to 470 nm light, while archaerhodopsin from Halorubum strain TP009 (ArchT) is an inhibitory proton pump that responds to 566 nm light (Yizhar, 2011a). An important difference is that, unlike excitatory opsins, inhibitory opsins requires constant light to maintain inhibition as the pump would stop shuttling protons or chloride ions. Behaviorally, researchers use excitatory opsins to cause gain-of-function and inhibitory opsins to cause loss-of function (Fenno, Yizhar and Deisseroth, 2011).

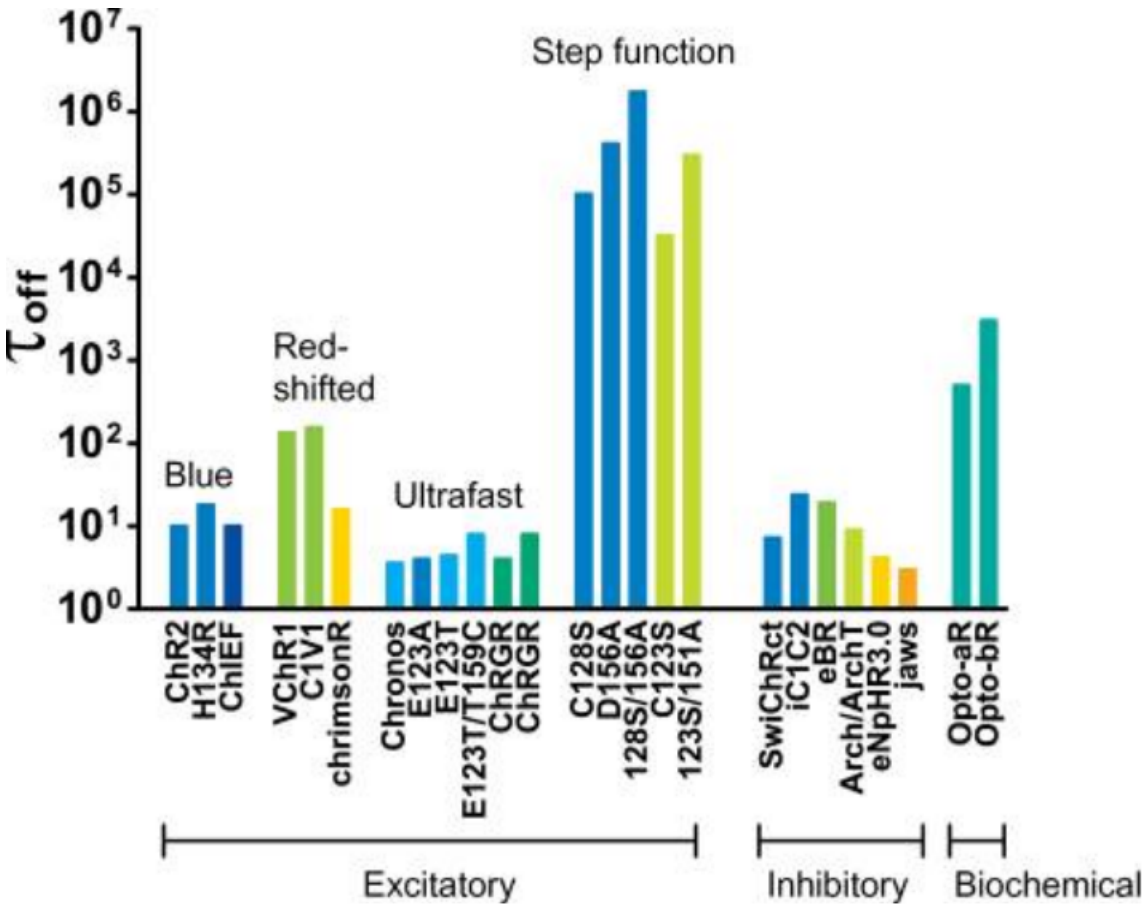

Figure 11. Graph of available opsins, their excitation wavelength and their deactivation time constant. τ_{off} indicates the opsin's deactivation time constant (i.e. fast opsins have smaller τ_{off} than slow opsins), while color indicates the optimal wavelength of light used to stimulate the opsin. (Taken from Guru et al., 2015)

Continued efforts to molecularly engineer opsins or to screen for novel opsins have produced many different opsins, each with their own distinct properties and advantages. For example, these efforts led to opsins that: responds to different wavelength of light (i.e. red-shifted opsins), have both a peak activation and inactivation wavelength (i.e. bistable or step-function), or have altered τ_{off} from milliseconds to 30 minutes (i.e. stable step-function opsin) (Berndt et al., 2008; Bamann et al., 2010; Yizhar, 2011b). Whether its modifying old opsins or finding novel ones, these efforts have led to increased efficiency and precision of opsin activity as well as adaptability of optogenetics to fit any experimental conditions (Figure 11).

Targeting opsins expression in adult-born neurons

Genetic targeting of opsin expression allows for the manipulation of specific neurons or neuronal populations. There are two major delivery methods to genetic targeting, viral vectors and transgenic animal lines, and they can be used to complement or in combination with one another. Viral expression of opsins is rapid, flexible, and very potent (i.e. high expression number). The most common viral vectors used to deliver opsins into neurons are lentiviral (LV) vectors and adeno-associated viral (AAV) vectors (Yizhar et al., 2011a). While RVs can also be used to deliver opsins, they very specifically infect actively-dividing cells, such as type II cells of the SGZ. Although researchers can achieve spatial restriction of opsin expression by intracranially injecting low volumes of the virus into a brain region, there are more sophisticated ways of conferring neuronal specificity to the delivery method.

Opsin-containing viral vectors can be used to target specific neuronal populations by altering their viral tropism (i.e. specificity of a virus for its host tissue) and/or the promoter associated with the opsin gene. Viral tropism of AAVs is determined by their serotype, each with their own distinct genome and capsid, while that of LVs is determined by the surface glycoproteins on its viral envelope which interacts with the cell surface receptor of target cells prior to its entry into the cell (Wu et al., 2006; Parr-Brownlie et al., 2015). Pseudotyping alters tropism by replacing AAV's capsid or LV's glycoprotein to one that better infects the target cell. In regards to studying neurogenesis, when LV is pseudotyped with Vesicular stomatitis Indiana virus' G protein (VSVg), it is effective at infecting cells in the SGZ and DG cell layers, while it more specifically infects mature granule cells when pseudotyped with murine leukemia virus glycoprotein (Watson et al., 2002).

The promoter controlling opsin expression can also further restrict where the opsin is expressed (Figure 13B). While promoters such as Elongation Factor 1 alpha (EF1α) is ubiquitous and leads to opsin expression in all infected cells, the nestin promoter will restrict opsin expression only to neural progenitor cells (Beech et al., 2004). Thus, a researcher aiming to infect type II cells in the SGZ of the DG could construct a VSVg pseudotyped LV containing an opsin under the control of a nestin promoter. It is important to note that viral vector capacity is limited as AAVs can carry a genome of up to 5 kb and LV 9 kb, which means that the full complement of enhancers and insulator elements in the promoter sequence is not included, causing reduced

specificity (for reference, the EF1α promoter in lentivirus vector is 1.2 kb) (Yizhar, et al., 2011a).

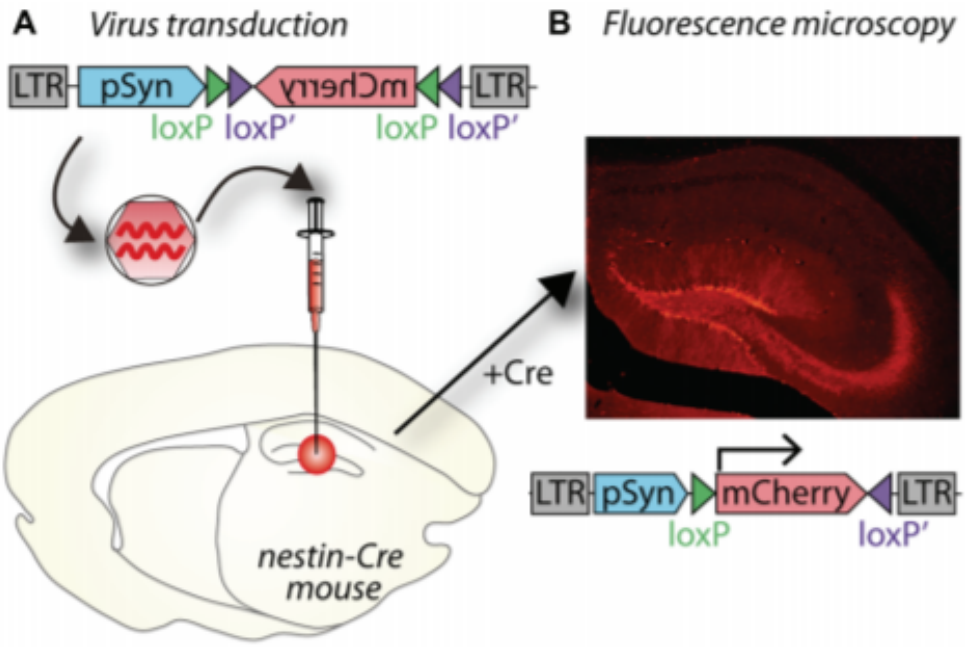

Figure 12. A sample Cre-lox system. (A) A LV containing a double floxed inverted mCherry, a fluorescent protein, gene downstream of a pSyn promoter is injected into the DG. (B) Cre inverts the mCherry gene, leading to its expression, as visualized by fluoresceence microscopy (Taken from Parr-Brownlie et al., 2015)

To enable the inclusion of the full promoter sequence and maximize specificity, a site-specific recombinase system called Cre-lox, combining viral vector delivery and transgenic mouse technology, is utilized (Nagy, 2000). The Cre-lox system deletes or inverts DNA sequences by taking advantage of the P1 bacteriophage-derived Cre protein's ability to recognize homotypic loxP recognition sites. Floxed DNA sites are either excised (loxP sites are in the same orientation) or inverted (loxP sites are in

opposite orientation) when exposed to the Cre protein (Araki, Araki and Yamamura, 1997; Sauer, 1998). By creating a transgenic Cre mouse with the full nestin promoter sequence and injecting it intracranially in the DG with a viral vector containing a floxed opsin sequence, neurogenesis researchers may express opsins in nestin+ type I and type II cells in the SGZ of the DG (Figures 4 & 12).

The two main transgenic Cre lines used in neurogenesis research are the nestin-based Cre line and the DCX-based Cre line, with the DCX line being used to study new neuron maturation as it avoids Cre expression in stem-cell populations (Figure 4). When crossed with a reporter line, they have been used to study the lineage of adult-born neurons in the DG, their maturation and their physiology (Fukuda et al. 2003; Zhang et al., 2010; Cheng et al, 2011). A reporter line is a transgenic lox line containing a floxed STOP cassette in between a reporter gene that codes for a fluorescent protein and a strong ubiquitous promoter (e.g. CAG or Rosa26). In a nestin Cre-lox reporter mouse, only when Cre is expressed does the STOP cassette get excised and the fluorescent protein expressed (Enikopolov et al., 2015). For this purely transgenic Cre-lox mouse system to be used in studying the function of adult-born neurons in learning and memory, a layer of temporal control must be added in order to express opsins in specific populations of new adult-born neurons.

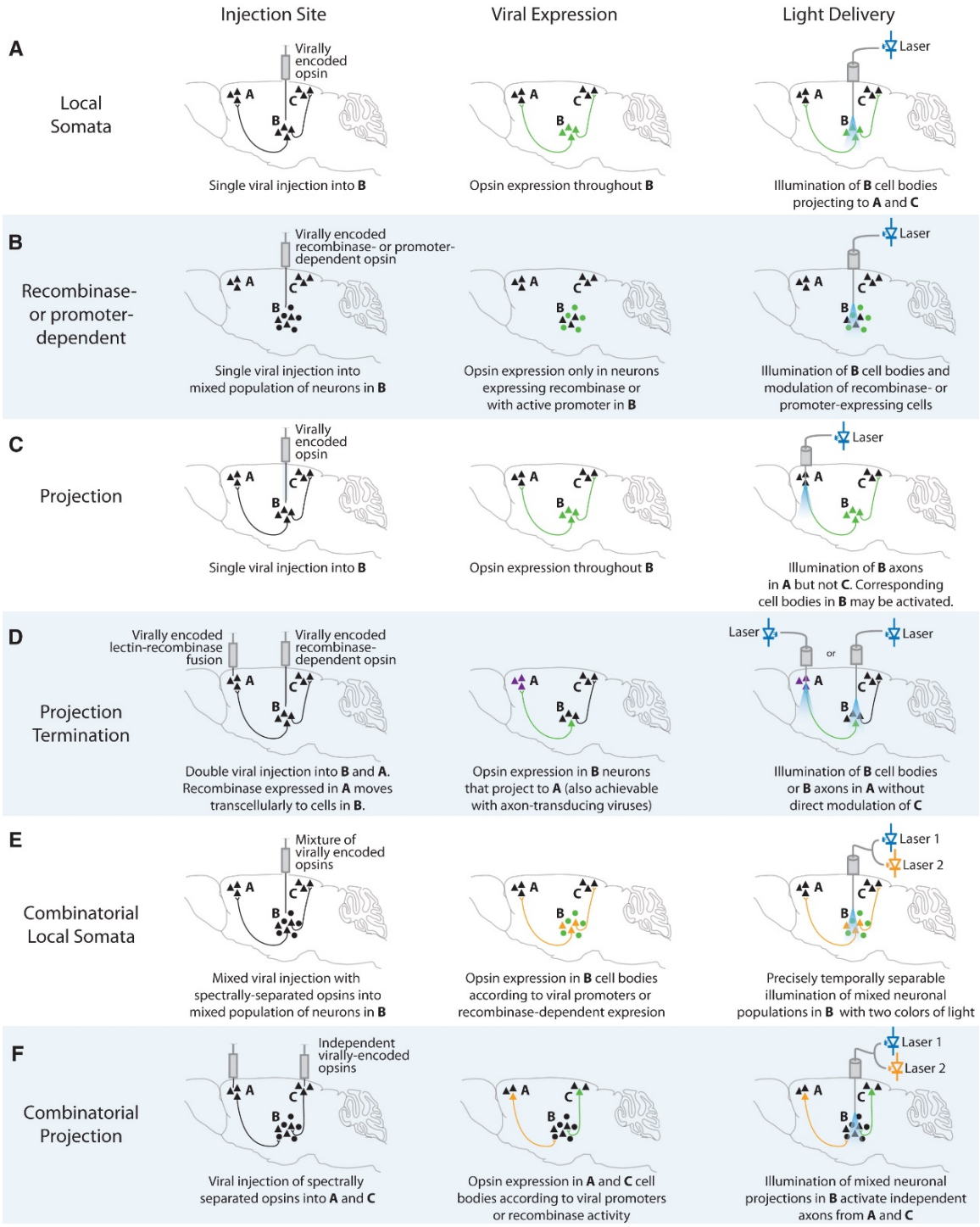

Figure 13. Targeting tools for optogenetics *in vivo*. (Taken from Yizhar et al., 2011a)

This temporal control can be found in inducible CreER lines. For example, a nestin CreER-lox line will only express the floxed gene in the presence of exogenous TM or, more specifically, its metabolite, 4-Hydroxytamoxifen (Metzger et al., 1995). TM-dependent expression of the Cre protein allows for time-specific labeling of populations of new neurons, similar to how viral vectors can only infect cells around the time of its injection. It is important to note that the overexpression of CreER protein will result in TM-independent expression of the opsin as CreER will be able to leak into the nucleus and interact with the STOP cassette (Chen et al., 2009), while underexpression of CreER will impair efficient TM-dependent expression of opsin. An experiment done by Sun et al. (2013) looking at reporter protein expression levels in three different nestin CreER^{T2} mice developed by different research groups has shown that there is a trade-off between efficiency and specificity of protein expression. Research should be done and caution taken when choosing the appropriate nestin CreER and opsin lox transgenic lines.

Light-delivery devices

Light-delivery technology is a necessity for optogenetics to work. *In vitro*, the opsins can be activated by mercury arc lamps, lasers, light-emitting diodes (LEDs), and LED arrays (more than one light source) (Boyden et al., 2005; Wang et al., 2009; Cardin et al., 2010; Steude, et al., 2016). *In vivo,* development in optics is crucial in order to safely deliver visible light at high intensity to the brain of freely moving mammals. Consider that the light delivered is 100 times greater than required by the opsin-expressing cells (~100 mW) to account for expected scattering losses over brain volume

(Deisseroth et al., 2007). Stimulating opsins in live animals during behavioral tasks can be done either with laser light delivered via optical fibers or with fiber-coupled LEDs (Aravanis et al., 2007; Wang et al., 2010). Unfortunately, both of these methods make use of tethered systems which require researchers to physically restrain animals during attachment and detachment of the optical fiber. In addition to giving the animals unwanted stress, they limit the experimental environment. Recent innovations have led to the creation of wireless implantable LEDs which uses either radio-frequency (RF) or near-field communications (NFC) hardware as a power source which only requires a single stereotaxic surgery to attach and allows for more experimental conditions (Montgomery et al., 2015; Shin et al., 2017) (Figure 14).

The use of fiber optics to deliver light allows for a novel technique called project targeting and for simultaneous readout of neuronal and projection activity during behavioral tasks (Deisseroth, 2015). Projection targeting refers to delivering light to a brain region where the axons of the opsin-expressing neurons reside. A typical projection-targeting experiment involves infection in one brain region by a viral vector containing an opsin with enhanced membrane-trafficking capabilities, followed by fiber optic light delivery to the brain region that the axon of the infected neurons project to (Deisseroth, 2014; Gunaydin et al., 2014) (Figure 13C,D). This technique separates the opsin-expressing cell body from potential heat damage and also avoids direct nonspecific excitation of the neural tissue. Simultaneous collection of neuronal activity information is also a major advantage of using fiber optics and this will be explored later in the discussion on the study done by Daniels et al. (2016)

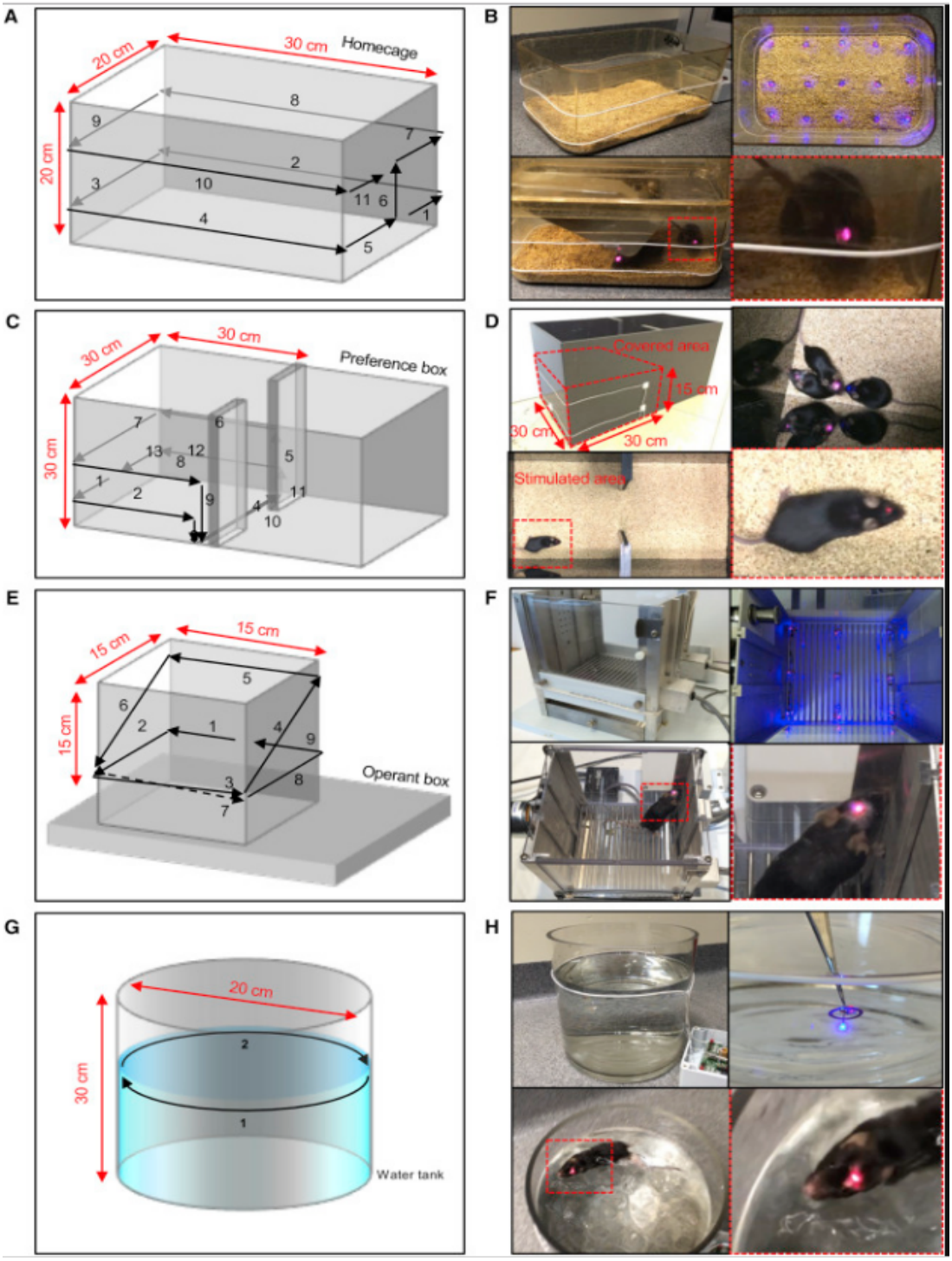

Figure 14. Wireless NFC device for optogenetics in various apparatus. The wireless device works in various environments, including (A) home cage, (C) real time place preference box, (E) operant conditioning box, and (G) a water tank (Taken from Shin et al., 2017)

STUDIES USING OPTOGENETICS TO EXPLORE NEUROGENESIS

In this section we will examine three studies demonstrating how optogenetics has been used to overcome limitations of past methods and further our understanding of adult hippocampal neurogenesis. Only results gathered by using optogenetics methods will be discussed.

Adult-born neurons form functional synapses with target cells

Around the time optogenetics was still in its early development, Toni et al. (2008) searched for evidence of functional synapses in adult-born neurons in the DG. Researchers know that these adult-born granule cells (abGCs) receive functional axo-somatic, axo-dendritic and axo-spinous inputs from entorhinal cortex and local inhibitory interneurons (Laplagne et al., 2006). It is also well-documented that during the survival phase, the granule cells elongate their axons to contact pyramidal cells in the CA3 region, while also making contact with inhibitory interneurons of the GCL, hilus and CA3 as well as excitatory mossy cells in the hilus (Henze Urban and Barrionuevo, 2000). However, due to technical challenges, there has not been data demonstrating functional output from the granule cells to these neurons. A possible method of measuring functional output would be to use a double patch recording of labelled granule cells to the target neurons in brain slices, but this would be a difficult undertaking as each granule cell only contacts a small number of potential neurons.

To overcome this difficulty, Toni et al (2008) used optogenetics to activate a large cohort of abGCs, which would increase the chances of finding and recording the activity

of connected target neurons. In one experiment, they infected 15 weeks-old mice with a replication-deficient retroviral vector containing either the ChR2-GFP transgene or the C-terminally truncated version of ChR2 fused to monomeric red fluorescent protein 1 (mRFP1) transgene driven by the constitutive promoters CAG and PGK-1 respectively. Three weeks post injection (wpi), brain slices from the mice were collected and prepared for electrophysiology and pharmacological treatments.

Using a Leica DMLS microscope, the researchers searched for ChR2-expressing cells (i.e. GFP+ or RFP+ cells) and made whole-cell patch-clamp recordings from neighboring cells in the GCL/hilar border, hilus and pyramidal layer. They recorded the target cells' postsynaptic membrane currents, while delivering 10ms pulses of blue light at 0.2-4 Hz to excite and activate the abGCs. As a result, they were able to successfully induce postsynaptic currents (PSCs) that were time locked to the activation of ChR2-expressing cells in 14 of the 107 potential target cells (i.e. 11/76 interneurons, 1/16 mossy cell, 1/11 CA3 pyramidal cell and 1/4 unclassified neuron). To reaffirm that the PSCs are evoked by the light-activated ChR2-expressing abGCs and to further characterize the nature of the synaptic transmission, the group treated the brain slices with three different drugs: 1) DCG-IV, a metabotropic GluR agonist that reduces neurotransmitter release at the presynaptic terminal, 2) kynurenic acid (Kyn), an AMPA/NMDA glutamate receptor antagonist, and 3) bicuculline methiodide (BMI), a $GABA_A$ receptor antagonist. They found that: 1) DCG-IV blocked PSCs (Figure 15a,b), 2) Kyn consistently reversibly abolished PSCs (Figure 15a-f), and 3) In some cases, BMI was able to block a substantial portion of PSCs on a pyramidal cell (Figure 15e,f).

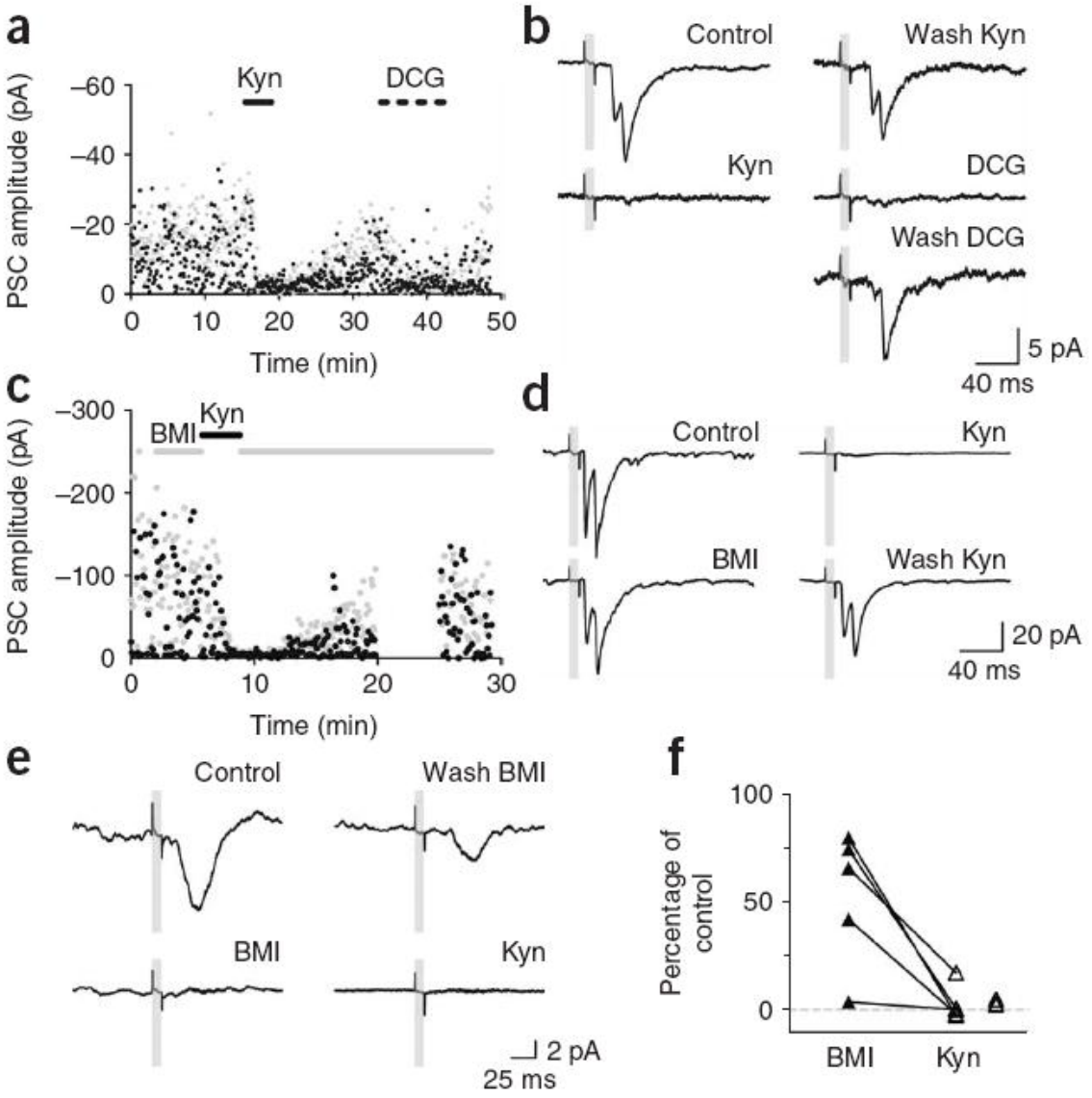

Figure 15. Pharmacological treatments by DCG-IV, Kyn, and BIM demonstrate glutamate release by adult-born granule cells. (a,b) Amplitudes and average amplitude of PSC peaks in hilar internurons during application of Kyn and DCG, (c,d) Amplitudes and average amplitude of PSC peaks in aGCL/hilar border interneuron during application of BMI and Kyn (acquisition of data was interrupted between 20-25 min), (e,f) Average amplitude of PSC peaks in a polysynaptic CA3 pyramidal neuron (Taken from Toni et al., 2008)

The results not only reaffirm that abGCs form functional contacts with target neurons, but also demonstrate that the contacts are glutamatergic synapses. BMI's blockage of PSCs does not contradict the glutamatergic nature of the granule cell's synapse. This finding is consistent with past studies showing the possible recruitment of GABAergic interneurons by DG cells to pass on its signal to CA3 neurons, which results in the formation of a polysynaptic pathway instead of a direct monosynaptic glutamatergic pathway (Scharfman, 1994; Scharfman, 1995).

Time-dependent role of adult-born neurons in memory retrieval

Having now known that abGCs form functional synapses with CA3 pyramidal neurons, Gu et al. (2012) sought to test if these projections play a role in learning and memory and, taking into account the unique physiological properties of immature neurons, if this role is time-dependent. Adult-born neurons have been known to display enhanced plasticity to inputs from surrounding cells and the entorhinal cortex between 4-6 weeks after birth, but it is unknown if the same enhanced plasticity can be found in its output synapses (Ge et al., 2007; Tashiro, Makino and Gage, 2007). Using retroviral delivery of a modified ChR2 opsin called ChIEF-dTomato, the researchers were able to induce LTP in differently-aged abGCs *in vivo* using a technique called theta-burst stimulation. They observed the amplitude of field excitatory postsynaptic potentials (fEPSPs) in the CA3 region before and after the TBS-induced LTP and found that the 3 and 4 wpi cohorts, but not the 8 wpi cohort, of granule cells showed enhanced plasticity in their output synapses (i.e. increased amplitude of fEPSP after LTP) (Parent et al., 1999). Additionally, they found that the potentiation disappeared when the mice were

given an intraperitoneal (i.p.) injection of a T-type Ca2+ channel blocker, mibefradil, providing strong evidence for the channel's involvement in the immature granule cells' enhanced plasticity (Schmidt-Hieber, Jonas and Bischofberger, 2004).

To study the effects of the 4 wpi granule cell's role in learning and memory, the researchers used optogenetics to temporarily, not permanently, inactivate different aged abGCs (i.e. 2, 4, and 8 wpi) during hippocampal-dependent Morris water maze (MWM) and fear conditioning behavioral paradigms. Prior to the behavioral tasks, they injected mice with a self-inactivating retrovirus containing the inhibitory opsin, Archaerhodopsin (Arch), into the hilus of their hippocampus to infect a cohort of newly-dividing cells in the SGZ. In order to reliably deliver light into the Arch+ cells, they implanted customized optrodes and delivered light through optic fibers via an optic-rotary joint.

The researchers first tested the effects of silencing 4 week-old abGCs on memory acquisition using the MWM. At 4 wpi, half of the mice were trained to find the hidden platform with the lights on (i.e. "light" group) while the other half with the lights off (i.e. "no light" group). The idea is that with the granule cells silenced, mice in the light group would learn to find the hidden platform slower than mice in the no light group. The results showed no difference in escape latency between the light and no light group across the training trials, which was sufficient data for the researchers to conclude that 4 week-old abGCs do not play a role in memory acquisition. Next, the researchers tested its potential role on memory retrieval. This time all the mice were trained with the lights off (i.e. abGCs still functional) but were split up into light and no light group during the first

probe test. For the second probe test, mice in the light group were put into the no light group and vice versa, thus each mouse served as its own control (i.e. within-subject design). Unlike training, the probe test does not have a platform and so memory retrieval is measured by the percentage of time the mice spent swimming in the quadrant where the platform used to be.

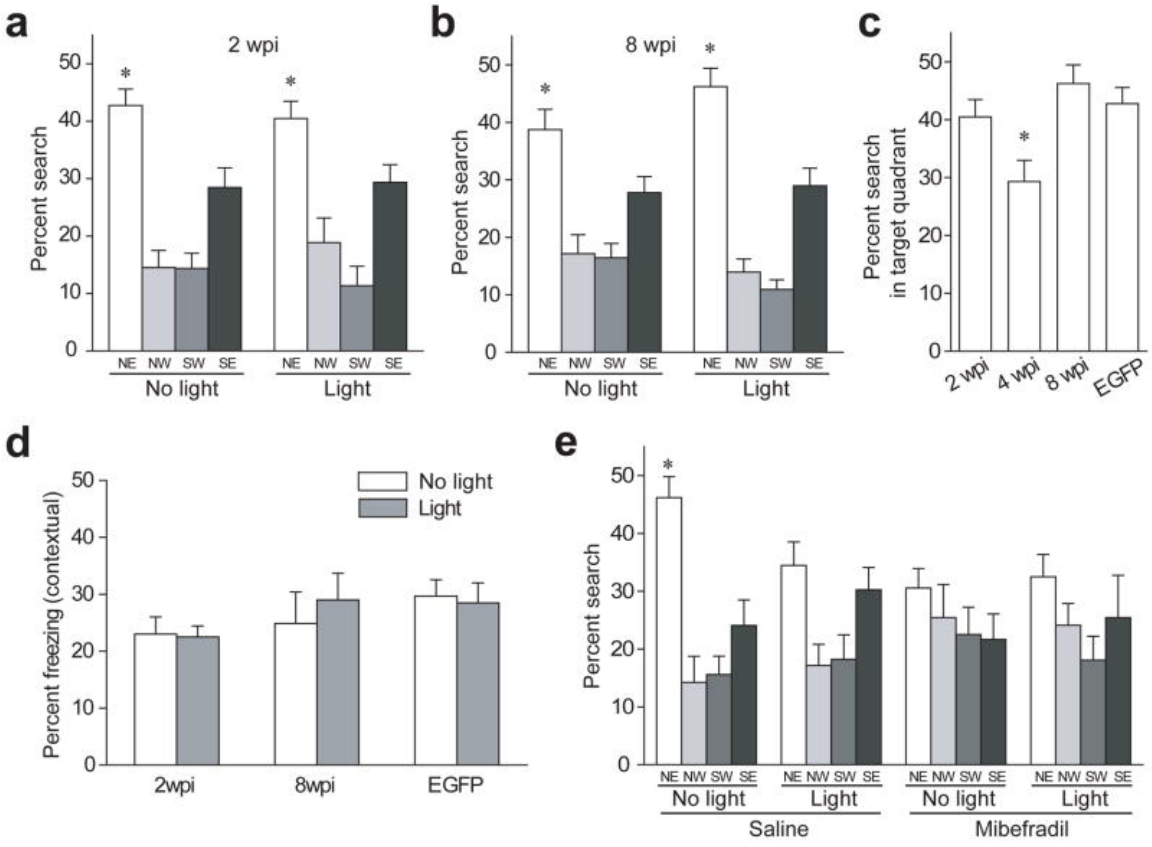

Figure 16. Age-sensitive role of adult-born neurons on hippocampal-dependent tasks. (a-b) Silencing abGCs at 2 or 8 wpi showed no effect on memory retrieval in MWM. (c) Silencing of 4 wpi group in MWM led to a significant reduction in time spent in searching in the target quadrant compared to 2 and 8 wpi groups, (d) Silencing of 2 wpi and 8 wpi abGCs during fear conditioning did not affect fear memory retrieval, (e) Mibefradil injection prevented hippocampal memory retrieval of 4 wpi animals during MWM probe test (Taken from Gu et al., 2012)

The results showed that the 4 wpi mice spent less time in the target quadrant during the probe test when the light is on. When this experiment was repeated on 2 and 8 wpi cohorts, they found no significant differences in the time spent in the target quadrant when the light is on vs when the light is off (Figure 16a-b). In fact, when comparing the percent of time spent in target quadrant across the differently-aged granule cells, the 4 wpi light group showed significant less time spent searching in the target quadrant compared to the 2 and 8 wpi light groups (Figure 16c). Lastly, i.p. injection of mibefradil to the 4 wpi no light group produced similar searching time in target quadrant when compared to the saline-injected light group (i.e. no mibefradil administration but adult-born neurons are silenced) and substantially decreased searching time compared to the saline-injected 4 wpi no light group (i.e. no mibefradil administration and adult-born neurons remain functional) (Figure 16e). Combined with the previous result which demonstrated T-type Ca2+ channel's importance in the immature granule cell's plasticity, these data suggest that the heightened plasticity of 4-weeks old granule cells plays a key role in increasing memory retrieval.

Impairments in memory retrieval is not specific to the MWM task as researchers tested 2, 4, and 8 wpi mice in a fear conditioning paradigm. The mice were trained with a single tone-shock pairing in a particular context and were tested in the same context either with lights on or lights off. They found that 4 wpi, not 2 nor 8 wpi, mice showed reduced freezing behavior (i.e. impaired retrieval of contextual fear memories) when their granule cells were temporarily silenced (Figure 16d). As expected, placing the mice in a different context and presenting them with the tone while silencing these cells did not

affect their freezing behavior as retrieval of tone does not depend on hippocampal functions (Kim and Fanselow, 1999). By using optogenetics to temporarily silence differently-aged granule cells, the researchers provided evidence to the time-dependent role of young adult-born neurons on memory retrieval and to the possible mechanism behind the phenomenon (i.e. increased T-type Ca2+ channels in 4-week old granule cells causes enhanced synaptic transmission).

Role of adult-born neurons in context discrimination

To test the hypothesis that adult neurogenesis is associated with pattern discrimination, Daniel et al. (2016) used a transgenic CreER^{T2}-lox mouse expressing the inhibitory opsin, Arch, to explore the potential role of adult-born granule cell on contextual encoding and discrimination. The group crossed a nestin CreERT2 mice with a lox mouse containing Arch-EGFP, creating a Nestin^{Arch} line. Adult Nestin^{Arch} mice were given TM at week 1 to allow for expression of Arch in nestin-expressing type 1 and type 2 cells in the SGZ. At week 4, they underwent targeted fiber optic implantation, allowing for the control of the Arch expressing neurons. By the time the animals were tested in the fear conditioning paradigm or pattern separation task, the Arch-expressing granule cells would be 6 weeks-old or younger (Figure 17A).

To examine effects on context encoding, Nestin^{Arch} and control mice with fiber optic implantation received targeted light illumination of the dorsal DG and were allowed to explore a conditioning context before receiving a shock. A day later, they were exposed to the same context in the absence of light and Nestin^{Arch} mice froze significantly

less than control mice, indicating the need for adult born granule cell activity for effective contextual encoding (Figure 17B). Next, they examined if the cells play a role in context discrimination. This experiment was done over 9 days. On day 0 the researchers allowed the mice to explore a conditioning context (A) before receiving a shock. Every subsequent day, the mice spend 2 hours in a similar but no shock context (B) before being put into context A and shocked again. There are 2 conditions to the experiment: 1) both control and Nestin^{Arch} mice receive light illumination when they are in context A or 2) when they are in context B (Figure 17C,D). The idea being that mice who learned to discriminate would display more freezing behavior in context A (shocked) than in context B (similar context but no shock).

The results show that Nestin^{Arch} mice in condition 1 (i.e. suppressed activity of abGCs while in shocking context A displayed intact context discrimination abilities, similar to the control mice, however, Nestin^{Arch} mice in condition 2 (i.e. suppressed activity of abGCs while in non-shocking context B had impaired development of a discriminatory response (Figure 17C,D). This indicated that functional abGCs are required to discriminate between ambiguous or similar contexts and likely played a role in context encoding as Nestin^{Arch} mice in condition 2 were unable to encode context B as the safe context.

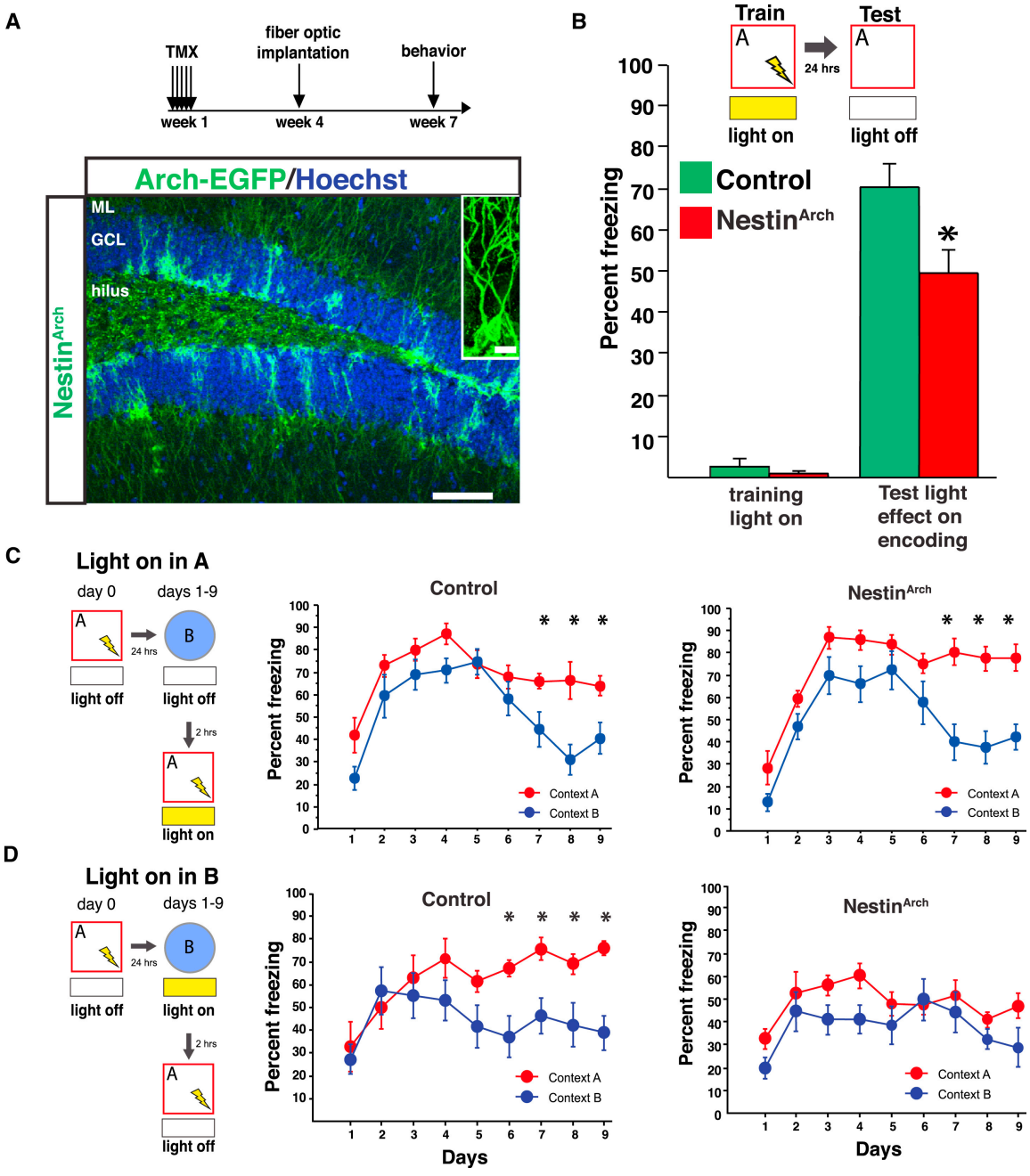

Figure 17. Silencing of abCGs impairs contextual encoding and discrimination. (A) Experimental design and sample expression of Arch in abGCs. (B) Silencing abGCs during training of fear conditioning impaired encoding of contextual fear. (C) abGC inhibition in the conditioning context did not impair contextual discrimination. (D) abGC inhibition in the non-conditioning context impaired contextual discrimination. (Taken from Daniel et al., 2016)

Although it wasn't reported, it would be interesting to see if there was a significant difference in freezing behavior between control and Nestin^{Arch} mice in condition 2 for the first two days. Reduced freezing behavior in Nestin^{Arch} mice in condition 1 would be further evidence as to the role of abGCs in contextual encoding. Having said that, any impairments in contextual encoding will be compensated by the reinforcing nature of the foot shocks, as seen in the increasing freezing behavior of Nestin^{Arch} mice in condition 1 when put in context A (Figure 17C right). In condition 2, the freezing behavior of Nestin^{Arch} mice actually decreased for both contexts A and B, which suggests that the mice are confused and cannot distinguish between which context is dangerous and which is safe. Overall, data obtained from these two experiments support the proposed role of abGCs in pattern separation, specifically in contextual encoding and discrimination (Clelland et al., 2009; Sahay et al., 2011, Nakashiba et al., 2012).

DISCUSSION

The papers discussed in the previous section have expanded our understanding of the role that abGCs play in learning and memory by using optogenetics to overcome limitations of past techniques. For example, due to the challenging nature of finding functional abGC-target neuron synapses for use in a double patch clamp recording experiment, Toni et al (2008) used optogenetics to activate a population of abGCs and increase his chances of finding and recording the activity of target neuron in response to output from abGCs. Neurogenesis studies using optogenetics to ablate neurogenesis during behavioral tasks points out that the reversible and temporary nature of these manipulations prevents compensatory changes from happening; changes that could have otherwise affected the results or the interpretation of the results. For instance, Singer et al (2011) conducted a long-term suppression of neurogenesis study using a nestin-tk mouse model and found that the lack of new neurons produced, as expected, significant deficits in DG LTP. Interestingly, the deficits in LTP were completely restored within 6 weeks without the presence of neurogenesis. Additionally, the researchers observed changes within the dynamics of the DG, namely an increase in the survival rate of newborn cells born immediately prior to the ablation and a reduction in inhibitory input to the granule cells of the DG. These changes to the structure and dynamics of the DG may have contributed to contradictory findings within the field (Shors et al., 2002; Winocur et al., 2006; Imayoshi et al., 2008; Deng et al, 2009; Deng et al, 2010).

However, no technique is perfect and therefore neurogenesis researchers must be mindful of the limitations and challenges of optogenetics when designing their

experiments. For example, in order to optogenetically silence abGCs during behavioral tasks and simulate their ablation, researchers would use inhibitory opsins such as Arch. Unlike excitatory opsins, inhibitory opsins require constant light delivery to maintain its inhibition on the neuron (Fenno, Yizhar, and Deisseroth, 2011). Considering that the power needed to penetrate 3 mm of non-human primate tissue and still activate the opsins is in the range of 200-500 mW/mm^2, prolonged inhibition may lead to the generation of excessive heat, which could damage the tissue surrounding the optic fiber. A recent study done by Senova et al., 2017 showed that rats receiving blue- (476 nm) or red-light (638 nm) stimulation at 200 mW/mm^2 and 40 Hz for 90 seconds did not show staining with TUNEL, a marker for cell apoptosis. More studies are needed to determine the safety parameters for laser use for prolonged neuronal inhibition. In addition to laser parameters, the target tissue element must be validated (e.g. by using fluorescent proteins) to determine the specificity of opsin expression and to validate the results of the experiment. This should be done regardless of the opsin delivery method, whether it be through viral vectors, by crossing transgenic lines, or by using recombination systems.

It is always important to consider the limitations and the known challenges of the different techniques when designing an experiment. Although optogenetics is still a developing technique, its ability to temporally manipulate specific neurons or cohort of neurons, along with its adaptable nature, makes it one of the most powerful tool for elucidating the role of adult-born neurons in the hippocampus.

CONCLUSION

Since the discovery of new neurons in the hippocampus in the 1960s, researchers have employed various methods and techniques to investigate the functional role of these new neurons. Among their many findings, they were able to trace the development of abGCs from NSCs, characterize the abGCs at different developmental stages, and associate its role in the brain with that of "pattern separation". However, due to various reasons, one being technical limitations, there exists contradictory findings as to the precise role played by these neurons in learning and memory. The development of optogenetics for use in neuroscience has allowed for reversible and temporally precise manipulations of abGC activity *in vivo*. Combined with other techniques and behavioral paradigms, researchers have been able to both reaffirm previous findings as well as gain new insights into mechanisms behind the proliferation, differentiation, and function of adult-born neurons. Future wave of discoveries within the field will depend on continued technological developments, the same ones which allowed for the acceptance of adult hippocampal neurogenesis in the 1980s and for the creation of optogenetics in 2005.

JOURNAL ABBREVIATIONS

Brain Res	Brain Research
Cell Res	Cell Research
Conf Proc IEEE Eng Med Biol Soc	Conference Proceedings: Annual International Conference of the IEEE Engineering in Medicine and Biology Society
Curr Biol	Current Biology: CB
Eur J Neurosci	European Journal of Neuroscience
Int J Cancer	International Journal of Cancer
J Biol Chem	Journal of Biological Chemistry
J Comp Neurol	Journal of Comparative Neurology
J Neural Eng	Journal of Neural Engineering
J Neurosci	Journal of Neuroscience
J Neurosci Methods	Journal of Neuroscience Methods
Mol Cell Neurosci	Molecular and Cellular Neurosciences
Nat Neurosci	Nature Neuroscience
Nat Protoc	Nature Protocols
Nat Rev Neurosci	Nature Reviews. Neuroscience
Proc Natl Acad Sci USA	Proceedings of the National Academy of Sciences of the United States of America

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